ISOLATION OF SNAKE VENOM TOXINS AND STUDY OF THEIR MECHANISM OF ACTION

Final Technical Report

by

Prof. André de Vries

January 1971

EUROPEAN RESEARCH OFFICE

United States Army

Contract Number DAJA 37-70-C-0447

Rogoff-Wellcome Medical Research Institute, Beilinson Hospital,

Petah Tikva, Israel

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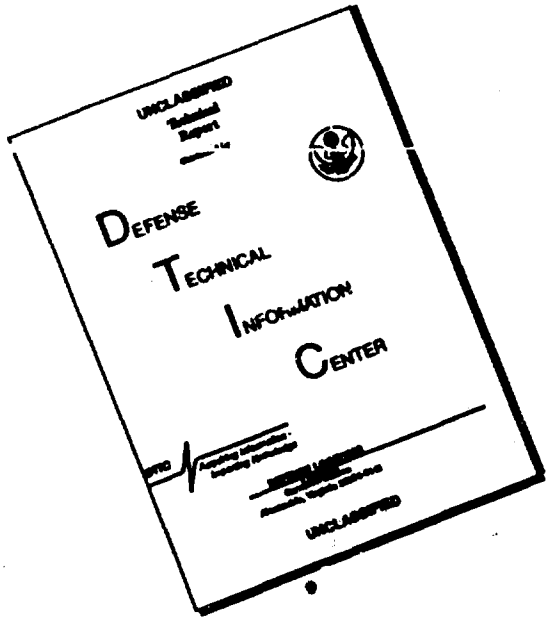

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13. ABSTRACT

1. A hemorrhagin from Echis coloratus venom, which appeared as a single protein in immunoelectrophoresis and in disc electrophoresis, possessed both hemorrhagic and proteolytic activities. Some of its antigenic determinants were identical to those of Vipera palestinae hemorrhagin. Partial cross neutralization of the lethal activity of V.p. hemorrhagin and of E.c. venom by heterologous antiserum was obtained. 2. Phospholipases A from the venoms of Naja naja and V.p. are able to hydrolyze lysolecithin at alkaline pH. 3. Haemachatus haemachatus (Ringhals cobra) phospholipase A hydrolyzes phospholipids of brain cell membranes and destroys part of the membranal system for histidine uptake. 4. A direct lytic factor from Ringhals venom, known to interact with unmodified red cell membranes, acts equally well on sialic-acid-depleted membranes. 5. E.c. venom administered intravenously causes damage to mouse brain capillary endothelial cells revealed by electron microscopy.

Key words:
Snake venom; Venom, snake; Hemorrhagins from snake venoms; Phospholipases from snake venoms; Lytic factor from snake venoms; Vipera palestinae; Naja naja; Echis coloratus; Cobra; Haemachatus haemachatus; Neurotoxin from snake venom

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Abstract:

The work reported herein is a continuation of our research on snake venoms and their toxins, done in previous years. It includes purification of an additional toxin - hemorrhagin from Echis colorata venom - and comparison of its antigenic properties to those of Vipera palestinae hemorrhagin, further characterization of phospholipases A derived from two different snake venoms, further studies of the action of phospholipase A on brain slices and of the action of a lytic factor from cobra venom on red blood cells, and study of a neurotoxic effect of the venom of Echis coloratus. The methods employed were: column chromatography, thin layer chromatography, electrophoresis, current immunological tests, extraction and analysis of phospholipids and electron microscopy. Experimental work in these various directions gave the following results: 1. A hemorrhagin from Echis coloratus venom, which appeared as a single protein in immunoelectrophoresis and in disc-electrophoresis, possessed both hemorrhagic and proteolytic activities. Some of its antigenic determinants were identical to those of Vipera palestinae hemorrhagin. Partial cross neutralization of the lethal activity of VP hemorrhagin and of EC venom

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by heterologous antiserum was obtained. 2. Phospholipases A from the venoms of Naja naja and Vipera palestinae are able to hydrolyze lysolecithin at alkaline pH. 3. Ringhals phospholipase A hydrolyzes phospholipids of brain cell membranes and destroys part of the membranal system for histidine uptake. 4. A direct lytic factor (DLF) from Ringhals (cobra) venom known to interact with unmodified red cell membranes, acts equally well on sialic acid-depleted membranes. 5. Intravenously administered Echis coloratus venom causes damage to mouse brain capiliary endothelial cells.

Table of Contents

Table of Contents

REPORT

Introduction

Snake venoms are complex mixtures of components with various activities. Our work in recent years has been directed towards the isolation of some components from snake venoms, their purification and study of their properties and activities in well defined systems. Specifically the following have been studied: phospholipases A, direct lytic factor (DLF), neurotoxin, hemorrhagins. While the general purpose of this work is achievement of a rational treatment of patients suffering from snake bite, study of the purified active factors has yielded interesting information in such remote fields as enzymology, immunology, membrane structure and function and brain physiology.

The studies reported herein are:

1. Immunochemical studies on snake venom hemorrhagins.
2. Effect of phospholipase A on histidine uptake by mouse brain slices.
3. The kinetics of lysolecithin hydrolysis by purified Naja naja and Vipera palestinae phospholipase A_2.
4. Action of cobra venom lytic factor on sialic acid-depleted erythrocytes and ghosts.
5. Electron microscopical study of neurotoxic effects of Echis coloratus venom in mice.

1. Immunochemical studies on snake venom hemorrhagins

Hemorrhage is a prominent clinical sign of Vipera palestinae (VP) bite (1). Purified VP hemorrhagic toxin was previously proved to be homogenous in immunodiffusion, immunoelectrophoresis, ultracentrifugal analysis and rechromatography (2). The purified preparation still possessed proteolytic activity which was found to be distinct from the hemorrhagic activity by applying protease inhibitors such as soy bean trypsin inhibitor or DFP.

The purpose of this investigation is to further clarify the interrelationship between the proteolytic and hemorrhagic activities by immunochemical methods. Moreover, it is of interest to study the antigenic relationship of hemorrhagic toxins derived from other Viperidae snake venoms in order to find a possible common molecular basis for this toxic activity. Attempts were therefore made to purify the hemorrhagin of Echis coloratus and compare its antigenic make-up to that of VP hemorrhagin.

Methods

VP hemorrhagin was purified as previously described (2). The hemorrhagic activity was determined in mice (2) and the proteolytic activity was tested on gelatin (3). Antiserum against VP hemorrhagin was prepared in rabbits, as described previously (4). Quantitative precipitin test was carried out according to Kabat (5) and the dissolution of the immune precipitate was performed according to Dandliker (6). EC hemorrhagin was isolated by chromatography of the whole venom on a DEAE-cellulose column equilibrated with 0.005M phosphate buffer pH 8.1. After washing with the same buffer, gradient elution from 0.005M phosphate buffer pH 8.1 to 0.2M phosphate buffer pH 6.9 was carried out in two successive steps. The hemorrhagin was further purified by filtration through Sephadex G-100 column eluted by 0.04M phosphate buffer pH 7.4, followed by chromatography on DEAE-Sephadex A-50 column, using gradient elution from 0.01M phosphate buffer pH 8.1 to 0.25M phosphate buffer pH 6.9.

Immunoelectrophoresis was carried out in 1% agar gel in 0.05M barbital buffer pH 8.2 and acrylamide gel electrophoresis was performed according to Davis (7).

Results and Discussion

A. Immunochemical studies on VP hemorrhagin

Specific antiserum prepared in rabbits against VP hemorrhagin neutralized the hemorrhagic activity only, and had no effect on its proteolytic activity. Precipitation of VP hemorrhagin by its specific antibodies at the equivalence zone of the precipitin curve revealed that the supernatant fluid, obtained after removing the immune precipitate, contained proteolytic activity. While the protease activity was completely recovered in the supernatant, it was devoid of hemorrhagic activity. The proteolytic enzyme in the supernatant might still be bound to antibodies in the form of soluble complexes. This possibility was excluded by applying anti-rabbit-γ-globulins which did not precipitate any protease-antibody complexes. Moreover it was shown for trypsin-antitrypsin system (8) and for ribonuclease-antiribonuclease system (9), that the mere formation of antigen-antibody complexes even in the soluble form caused inhibition of the enzymatic activity. The above finding thus suggested that the hemorrhagic and proteolytic activities were associated with two different molecules.

The immune precipitate obtained at the zone of maximum precipitation was devoid of both proteolytic and hemorrhagic activities. Attempts were made to isolate hemorrhagin from the immune precipitate by its dissolution at high ionic strength 3M NaSCN at neutral pH. The dissolved precipitate was applied to a column of Sephadex G-100. The elution pattern provided evidence for dissociation of the precipitate into smaller antigen-antibody complexes, all of which were likewise inactive.

B. Purification of Echis coloratus (EC) hemorrhagin.

The hemorrhagin from EC venom was isolated by the following procedure. Ion-exchange chromatography of whole EC venom, carried out as described in Methods, yielded five protein peaks; the hemorrhagic activity was distributed between peaks 2, 3 and 4. Fraction 4, representing 6.8% of the total protein, was further purified by gel filtration through Sephadex G-100, yielding two protein fractions. The first fraction which possessed both hemorrhagic and proteolytic activities, was rechromatographed on DEAE Sephadex A-50. Two protein fractions were obtained; both fractions possessed proteolytic activity, whereas only the first one possessed hemorrhagic activity. This hemorrhagic fraction exhibited

one precipitin arc in agar immunoelectrophoresis and one protein band in acrylamide gel electrophoresis. The last step of the purification procedure reduced the specific toxic activity about two-fold. The lethal dose of the purified fraction was 24 µg per mouse (12 gr) by the intravenous route, as compared to 10.5 µg of the hemorrhagic fraction eluted from Sephadex G-100 and 11.5 µg of the whole venom.

Purified EC hemorrhagin exhibited antigenic determinants identical with those of purified VP hemorrhagin, when tested in immunodiffusion against specific anti-EC venom. However, VP hemorrhagin exhibited only partial antigenic identity with isolated EC hemorrhagin when tested against specific anti-VP hemorrhagin.

When tested for cross neutralization of lethal activity, it was found that anti-VP purified hemorrhagin neutralized 29% of the heterologous EC venom, while anti-EC venom neutralized 18% of the heterologous VP hemorrhagic fraction (Table 1). These results suggest that VP and EC hemorrhagins share antigenic determinants and therefore may have identical structure at certain parts of the molecule. The cross neutralization of the lethal activity suggests that part of the antibodies elicited were directed against the active site of the molecule which is identical in both VP and EC hemorrhag[illegible].

Table 1. Comparative neutralization of homologous and heterologous venom antiserum reactions.

Venom	Vipera palestinae hemorrhagic fraction	E. coloratus	E. coloratus	V. palestinae hemorrhagic fraction
Antiserum	Vipera palestinae hemorrhagic fraction	V. palestinae hemorrhagic fraction	E. coloratus	E. coloratus
Mouse LD_{50} neutralized/ml	360	102	390	70
Percent neutralization	100	29	100	18

2. Effect of phospholipase A on histidine uptake by mouse brain slices (shortened version from paper accepted for publication in Biochimica Biophysica Acta, 1971).

Cobra phospholipase A is known to interact with various biological membranes, bringing about hydrolysis of their major phospholipids. We have previously studied effects of phospholipase A treatment on a brain microsomal activity, Na^+, K^+ - dependent ATPase. Another membranal function - amino acid transport - has now been studied for its dependence on phospholipid.

Active transport of ions through membranes has been shown to depend on their phospholipids. Martonosi (1) has reported inhibition of Ca^{++} uptake by fragmented sarcoplasmic reticulum treated with phospholipase C, and Larsen and Wolff (2) have shown that the same enzyme inhibits uptake of iodide by thyroid slices. In the latter system no effect could be obtained with phospholipase A. We have studied the effect of cobra phospholipase A treatment on uptake and influx of histidine into mouse brain slices. Phospholipid content of the treated brain slices has also been determined.

The enzyme (phosphatide acylhydrolase, EC 3.1.1.4) was isolated from the venom of Hemachatus haemachates by paper electrophoresis and purified by gel filtration of a 4% trichloroacetic acid precipitate (3). It electrophoresed as a single band on a polyacrylamide gel.

Methods

Brain slices. Male white mice of a local strain, weighing 20-22 g, were used throughout. The mice were killed by decapitation. The brains were rinsed in saline and after removal of the brain stem cut transversely into thin slices with a razor blade. Routinely, 12 slices were obtained from each brain, having a total weight of 0.375± 0.003 g(mean ± S.E.; 15 determinations).

Histidine uptake. Slices obtained from one brain were suspended in 10 ml of Krebs-Ringer bicarbonate buffer containing 0.3% glucose and (^{14}C) histidine. The suspension was gassed with 95% O_2 - 5% CO_2, and incubated at 37°. At the end of incubation period the suspension was filtered and the slices homogenized in 2 ml of cold 5% trichloroacetic acid. After centrifugation 1 ml of clear supernatant was mixed with 5 ml Bray solution (4) and counted in a Packard liquid scintillation spectrometer with an efficiency of 52%.

In control experiments radioactivity in the medium at the end of the incubation period was also determined. Under the standard conditions described, the ratio of radioactivity in the trichloroacetic acid-soluble material obtained from 1 g brain (calculated) to radioactivity in 1 ml medium at 60 min was 7.82±0.36 (mean ± S.E.; 8 determinations). Other control experiments showed that 96% of the radioactivity in the slices was in the trichloroacetic acid-soluble material and that 95% of the radioactivity in this fraction chromatographed as histidine.

Lipid extraction and analysis. Brain lipid was extracted by chloroform - methanol (2:1, by vol). Phospholipids were separated on *Silica gel* G plates by two dimensional chromatography, using chloroform-methanol-water (65:25:4 , by vol.) as the first solvent system and 1-butanol-acetic acid-water (60:20:20, by vol.) as the second. Phosphorus in phospholipid spots was determined as described by Rouser and Fleischer (5).

Results and discussion

The effect of phospholipase A on histidine uptake from various concentrations of the amino acid is shown in Fig. 1. At 10 ug/ml the enzyme inhibited uptake from a wide range of

histidine concentrations (0.25-20 mM) to about the same extent, causing a decrease of 50-60% in the amount accumulated in brain slices on 60 min incubation. The run of the lower curve seems to show that treatment of brain cell membranes with phospholipase A destroys part of the saturable system for histidine uptake. Since inhibition in uptake of histidine could be attributed to the action of free fatty acids or the lysocompounds formed, control experiments were done with these compounds in the medium. Palmitic, lauric, and oleic acid, each tested separately at concentrations up to 1 mM, and lysolecithin at concentrations up to 400 µg/ml did not affect uptake of histidine under standard conditions. It therefore appears that impairment of histidine uptake by phospholipase A was a result of degradation of membrane phospholipids per sé.

Initial influx of histidine into brain slices preincubated with phospholipase A and then transferred into a C^{14}-histidine containing medium is presented by the curves in Fig. 2. Influx of the amino acid was inhibited in all pretreated brain samples to an extent that depended on enzyme concentration.

Phospholipid content of brain slices treated with phospholipase A in the medium used for uptake experiments has been determined. The results summarized in Table 2 show that hydrolysis of both lecithin and phosphatidylethanolamine depended on enzyme concentration and on the incubation period.

We conclude that a purified preparation of phospholipase A hydrolyzed the major phospholipid components of brain membranes. Histidine transport into membranes with reduced lecithin and phosphatidylethanolamine content is severely damaged. The damaged transport is at least partly due to inhibition of histidine influx.

Table 2

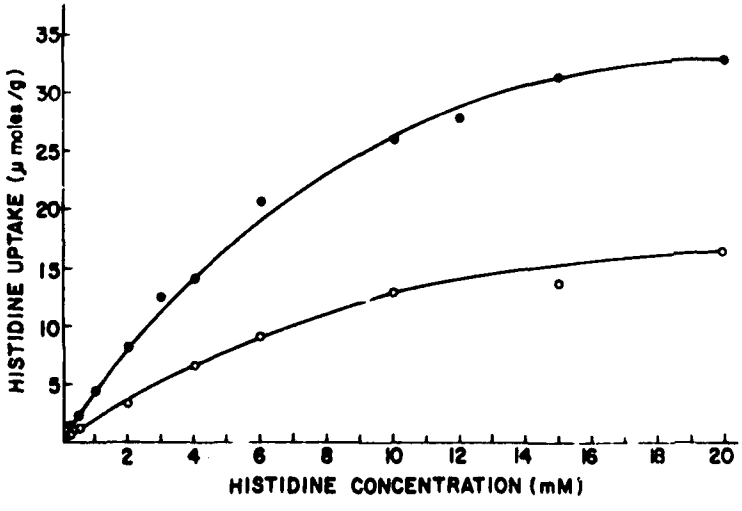
Hydrolysis of brain phospholipids by phospholipase A

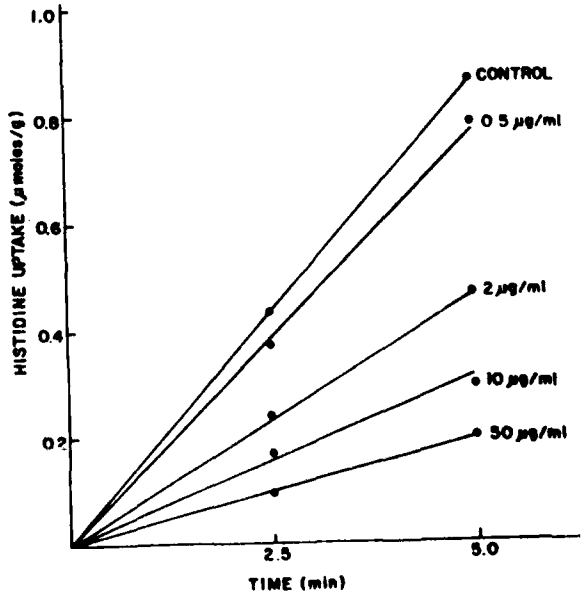
Enzyme concentration (μg/ml	Time of incubation (min)	Percent hydrolyzed	
		Phosphatidyl-ethanolamine	Phosphatidyl-choline
0	60	0	0
10	20	10	12.5
10	60	28	22
20	60	--	32

Figures

Fig. 1. Effect of phospholipase A on histidine uptake from varying initial concentrations of histidine. Brain slices were incubated with or without the enzyme for 60 min. Specific activity of histidine 1.6 μC/mmole ●——● without enzyme; ○——○ with phospholipase A, 10 μg/ml.

Fig. 2. Initial influx of histidine into phospholipase-treated brain slices. Slices were incubated for 60 min. in KR medium containing varying concentrations of phospholipase A. After being rinsed with saline the slices were introduced into the standard medium for uptake. Specific activity of histidine 16 μC/mmole.





3. The kinetics of lysolecithin hydrolysis by purified Naja naja and Vipera palestinae phospholipase A_2*

Phospholipases, the enzymes which release fatty acids from glycero-phosphatides, are classified into two kinds - A and B, according to whether they act on diacyl or monoacyl substrates, respectively. Phospholipases of type A catalyse the hydrolysis of one ester bond in 1,2 diacyl-sn-glycero-3-phosphatide forming a lysoderivative and releasing a free fatty acid. These enzymes have positional specificity, those hydrolysing the 1 (or α) position being designated as A_1, and those hydrolysing the 2 (or β) position as A_2. The snake phospholipases of type A investigated sofar are believed to be A_2 enzymes. Van Deenen and de Haas (1) found, using synthetic substrates, that whole Crotalus adamanteus (CA) venom catalyses the hydrolysis of both 1,2 diacyl-sn-glycero-3 phosphatide and 2-monoacyl-sn-glycero-3-phosphatide (the β - acyl-lysoderivative) whereas the 1 monoacyl-sn-glycero-3-phosphatide (the α-acyl-lyso-derivative) was not susceptible to the venom.

In the present study it is demonstrated that purified phospholipases of type A from the venoms Vipera palestinae (VP) and cobra Naja naja (NN) exhibit also phospholipase B activity hydrolysing the 1-monoacyl-sn-glycero-3-phosphatide (the α-acyl-lyso-derivative).

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*Submitted for publication in the Journal of Lipid Research.

Methods

Enzymes: The freeze-dried Vipera palestinae venom (obtained from the Department of Zoology, Tel Aviv University) was dialyzed against phosphate buffer pH 8.2 0.005M and chromatographed on DEAE cellulose. Elution was carried out with the above buffer and then with a pH 6.8 phosphate buffer in a gradient between 0.005M and 0.2M. The active fraction was rechromatographed on Sephadex G-50 using ammonium bicarbonate pH 7.2 0.1M as eluent. After lyophilization the fraction was homogeneous on acrylamide gel electrophoresis in acid and alkaline pH. The freeze-dried cobra Naja naja venom (L. Light and Co. Ltd. Colnbrook, England) was dialyzed against 0.005M ammonium acetate buffer, pH 6.0. The material was then chromatographed on CM-cellulose, with the above buffer, in a linear gradient between 0.005M - 0.5M. The fraction which emerged at the initial ionic strength and contained the main isoenzyme of the phospholipase A complex (2) was collected, lyophilized and rechromatographed on Sephadex G-50 as described above. The fraction thus obtained was homogeneous on acrylamide gel electrophoresis in acid and alkaline pH.

Substrates: Lecithin was isolated from rat liver extract, using adsorption chromatography on alumina (3). The fraction thus obtained was further purified by preparative TLC (4).

After elution from the plate, filtration through sintered glass filter and drying with nitrogen the material was dissolved in chloroform methanol 2:1 and kept at -20°C until use. Lysolecithin was prepared enzymatically from the purified lecithin by phospholipase A from various venoms. The crude preparation was further purified by preparative TLC analogous to the procedure with lecithin.

Conditions for enzymatic hydrolysis

The lipid substrates were prepared by drying the chloroform-methanol solutions of the phospholipid with nitrogen. 0.25 mM solutions in 0.1M ammonium acetate buffer pH 8.5 and pH 10.0 for lecithin and lysolecithin respectively were used. The media contained also 0.5 mM $CaCl_2$. Prior to incubation with the enzyme sonication was performed in lecithin containing systems. With lysolecithin this treatment was omitted, since 0.25 mM solutions were completely clear. The mixtures were incubated at 37° for a given time. After incubation the reaction was stopped by addition of methanol, the mixture was then evaporated with nitrogen and reextracted with chloroform methanol 2:1. The reaction products were chromatographed by one dimensional TLC. In a system of chloroform:methanol+10% ammonia 65:35:7.5 glycerylphosphoryl-choline (GPC) remained at the application point and lysolecithin and lecithin spots showed R_F values of 0.18 and 0.35 respectively. The percent hydrolysis was calculated from the phosphorus content of lecithin, lysolecithin and GPC containing spots.

Results and Discussion

In addition to their main phospholipase A activity the purified Vipera palestinae and Naja naja phospholipases exhibited pronounced phospholipase B activity, producing glycerylphosphorylcholine from lysolecithin under appropriate conditions of pH and reactant concentration. The phospholipase B activity was observed, whether the lysolecithin was prepared by the action of Vipera palestinae and Naja naja phospholipases or by Crotalus adamenteus venom (Table 3). Lysolecithin prepared by CA venom was shown to be the 1-monoacyl sn-glycero-3-phosphorylcholine (1). The lysolecithin used in the present study (prepared by purified V-P or NN phospholipases) behaved similarly to the lysolecithin obtained by CA and was assumed to have the same structure. The influence of pH on the activity of VP and NN phospholipases towards lysolecithin was similar. The activities remained rather low between pH 6.0-8.5 and then increased up to pH 10.5 (Fig. 3). The reaction velocity at about 30% substrate hydrolysis was proportional to the enzyme concentration. The Michaelis-Menten constants and Kcat values at pH 10 for the two enzymes are quite similar. For VP phospholipase K_m= 1.1 mM and Kcat = 0.45 sec^{-1} and for NN phospholipase Km_1= 1.1 mM and Kcat 0.9 sec^{-1} (Fig 4). The Kcat values are based on molecular weight of 20.000 and 15.000 respectively). The purified enzymes exhibit marked

differences in pH profile and activity when acting upon lecithin as compared with lysolecithin. The hydrolysis on lecithin has an optimum at pH 9 whereas that of lysolecithin increased beyond pH 10 without showing a maximum (Fig. 3).

Both enzymes were much more active towards lecithin than towards lysolecithin. With VP the activity towards lecithin was $5x10^2$ times higher than towards lysolecithin and with NN this ratio was about $3x10^5$. It seems likely that this could be due to the difference in the physicochemical properties of lecithin and lysolecithin micelles in water (5). It is worthwhile to emphasize that, whereas the activities of the two venom phospholipases towards lysolecithin were similar, the difference in their activities towards lecithin is rather large. The strong tendency of lecithin molecular dispersed in water to aggregate into bimolecular sheets and the difference in the activity of the VP and NN phospholipases to penetrate into a membranal structure (6,7,8) may be relevant.

Similarly to the phospholipase A_2 activity the phospholipase B activity of the purified enzymes was little effected by boiling at pH 5.5. Ca^{++} ions had a marked stimulatory effect and EDTA inhibited the hydrolysis completely. These latter observations

and the homogeneity of the phospholipase preparations are in favour of the assumption, that the purified phospholipases of VP and NN venoms are enzymes with dual activity, acting upon lecithin and lysolecithin as well.

Table 3

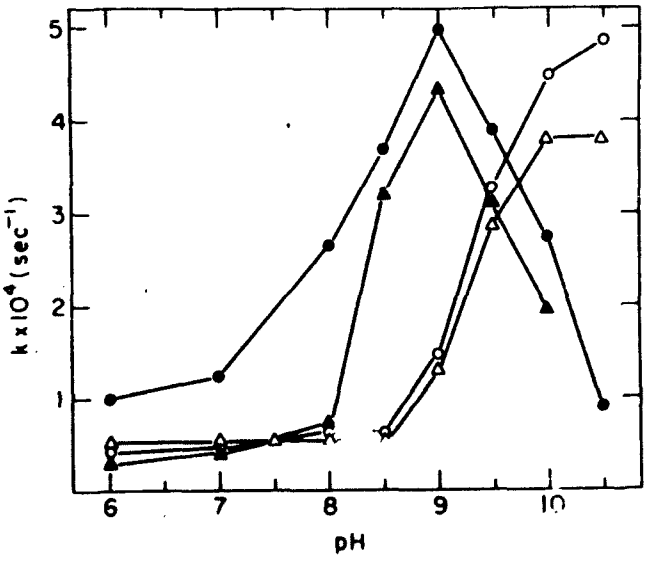
Phospholipase B activity of snake venom and purified enzymes on variously prepared lysolecithins

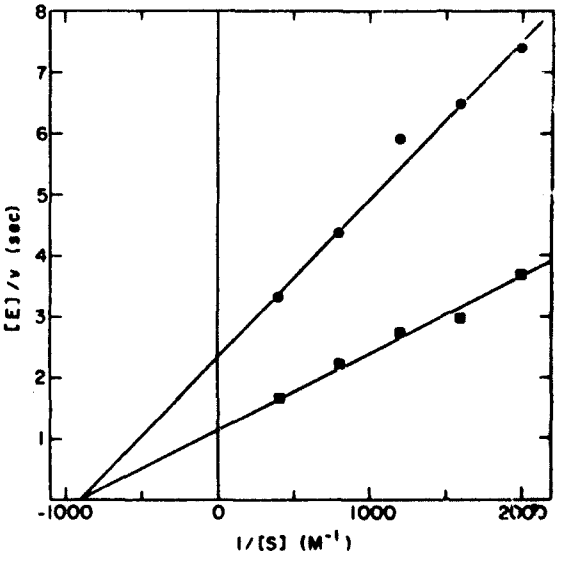
Phospholipase used for preparation of lysolecithin	Phospholipase B activity: Source	Conc. µg/ml	pH	Substrate hydrolyzed: Per cent (1 hr)
Naja naja (NN) purified	CA whole	450	7.0	15.0
			10.0	90.1
Vipera palestinae (VP) purified	CA whole	450	7.0	15.6
			10.0	80.0
Crotalus adamentaus (CA) whole	CA whole	450	7.0	27.0
			10.0	100.0
	NN purified	12	7.0	28.6
			10.0	93.0
	VP purified	25	7.0	13.5
			10.0	92.0

Figures

Fig. 3. Effect of pH on pseudo first order rate of hydrolysis of lecithin (full symbols) and lysolecithin (empty symbols) by phospholipase of Naja naja (triangles) and of Vipera palestinae (circles). The system contained 0.25 mM substrate in ammonium acetate buffer 0.1M, and mM $CaCl_2$. Enzyme concentrations: △ 7.0 µg/ml; ○ 14 µg/ml; ▲ 10^{-5} µg/ml; ● 10^{-2} µg/ml.

Fig. 4. Lineweaver - Burk plots for the hydrolysis of lysolecithin by the phospholipases of Naja naja (■) and Vipera palestinae (●).





4. Action of cobra venom lytic factor on sialic acid - depleted erythrocytes and ghosts. (published in Naunyn-Schmiedebergs Archiv f. Pharmakologie, 268:458, 1971.)

A basic protein isolated from cobra venoms, designated direct lytic factor (DLF), produces mild hemolysis in human erythrocytes and acts synergistically with snake venom phospholipase A (phosphatide acyl-hydrolase EC 3.1.1.4) enabling the enzyme to hydrolyze phospholipids in intact erythrocytes with ensuing strong hemolysis, and to hydrolyze phospholipids in red cell osmotic ghosts (1). Since Ca^{2+} was found able to replace DLF in the promotion of erythrocyte membrane phospholipid splitting by the phospholipase A, a possible similarity in the mode of action of DLF and Ca^{2+}, i.e. mediation of binding of the enzyme to negatively charged sites on the red cell surface, has been suggested (2). We therefore studied the effect of DLF on erythrocyte membranes from which sialic acids, the main source of the negative membrane charges, had been removed.

Materials and Methods

Venom fractions. Vipera palestinae and Ringhals (Haemachatus haemachatus) venoms were fractionated and the DLF and phospholipase A fractions purified as described previously (2).

Erythrocytes and erythrocyte ghosts. Washed erythrocytes obtained from freshly drawn normal human blood were used (1). Erythrocyte ghosts were prepared by hemolysis in hypotonic 0.01M Tris-HCl buffer (pH 7.2) containing 5 mM EDTA, and washed free of hemoglobin in the same buffer with EDTA omitted. Tris buffer made isotonic with NaCl was used for the last washing.

Neuraminidase (RDE) and trypsin treatment. 0.5 ml packed erythrocytes or their equivalent number of ghosts were suspended in 2 ml of physiological saline buffered with 0.01M phosphate (pH 6.4) and incubated with 25 µg RDE (Sigma, type VI), or suspended in 1.5 ml of phosphate buffered saline (pH 7.7) and incubated with 0.25 mg trypsin (Sigma, Type I) as described by Winzler et al. (3). After shaking for 1 hr at 37°C, the suspensions were sedimented and the sialic acids contents of the sediments and supernatants assayed by the thiobarbituric acid method of Warren (4). Neuraminidase treatment, which removed 84.8%±8.88

(mean of 9 experiments) of the erythrocyte and ghosts sialic acids, did not affect their phospholipid content and distribution (normal phospholipid values, see ref. 2). The same holds true for the treatment with trypsin, which removed 54.0%±5.38 (mean of 11 experiments) of membrane sialic acids as sialoproteins.

Assay of hemolytic and phospholipid splitting activities. Untreated as well as RDE- or trypsin-treated erythrocytes and ghosts were washed in physiological saline buffered with 0.01 M Tris- HCl (pH 7.2) and suspended in saline buffered with 0.1 M Tris at the same pH. 1.5 ml suspension containing 0.5 ml packed erythrocytes or their equivalent number of ghosts, venom phospholipase A and DLF in the amounts indicated in each experiment, was incubated for 2 hr at 37°C while shaking. Hemolysis was measured by determining the amount of released hemoglobin by the benzidine method (5). For determination of phospholipid splitting in erythrocytes and ghosts, the lipids were extracted, separated by thin-layer chromatography and estimated by their phosphorus content as described in detail elsewhere (2).

Results and Discussion

The degree of lysis produced in washed erythrocytes by DLF (maximum 5%), by Ringhals phospholipase (maximum 1%), or by the two in combination (maximum 90%) was not changed by pretreatment of the erythrocytes with neuraminidase or trypsin. Furthermore, treatment of the erythrocytes by neuraminidase or trypsin did not affect their susceptibility to the action of Ringhals phospholipase or to the enhanced action of Ringhals phospholipase and DLF combined, as evidenced by the respective degrees of splitting of phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl choline (Table 4). Similar results were obtained when osmotic ghosts were subjected to the action of Vipera palestinae alone or in combination with DLF.

The above results show that the action of cobra DLF on the erythrocyte membrane, producing direct hemolysis as well as enhanced phospholipid hydrolysis and hemolysis in the presence of phospholipase A, is not affected by removal of sialic acids or sialoproteins from the membrane. Thus, the negative charges contributed by the sialic acids are not required for the action of DLF on the erythrocyte membrane.

On the other hand, the possibility of attachment of DLF to acidic erythrocyte membrane proteins or to phospholipids or an interaction of DLF with SH-groups in the membrane, as postulated by Vogt et al. (6), remains open. Furthermore, since removal of the sialic acids and sialoproteins does not modify the availability of erythrocyte phospholipids to hydrolysis by phospholipase A alone, these components of the membrane surface appear not to be responsible for the low availability of the phospholipids in erythrocytes and their ghosts to the action of venom phospholipases.

Table 4. Synergistic effect of DLF and phospholipases on sialic acid-depleted erythrocytes and ghosts

Erythrocytes					Ghosts				
		Phospholipid splitting %					Phospholipid splitting %		
Pre treatment	Treatment	PE	PS	PC	Pre treatment	Treatment	PE	PS	PC
None	Ringh.* (3)	40.7	21.8	70.5	None	V.p. (9)	34.9±6.9	39.4±10.5	41.8± 8.9
	Ringh.*+DLF (4)	99.3	94.6	100.0		V.p.+DLF(6)	60.9±11.9	94.1± 5.1	80.8±10.1
RDE	Ringh.* (4)	39.7	22.7	70.3	RDE	V.p. (7)	41.3±18.7	54.0±26.1	46.2±19.2
	Ringh.*+DLF (4)	96.5	87.5	100.0		V.p.+DLF(6)	67.7± 8.7	96.4± 2.2	87.9± 5.4
None	Ringh.** (7)	12.7±4.8	10.4±9.0	32.3±11.3					
	Ringh.**+DLF (4)	96.9	80.8	95.2					
Trypsin	Ringh.** (6)	10.6±8.6	6.1±4.2	33.9±19.9	Trypsin	V.p. (3)	29.8	41.0	42.7
	Ringh.**+DLF(5)	94.3	91.2	99.1		V.p.+DLF(8)	63.4±14.5	95.4± 5.1	88.6±10.6

Erythrocytes and ghosts were incubated with venom fractions for 2 hr at 37°C as described in Methods. DLF, 600 ug; Vipera palestinae, 150 ug; *Ringhals phospholipase, 400 ug. **Ringhals phospholipase, 300 ug. Number of experiments in each group is indicated in parenthesis.

Abbreviations: Ringh., Ringhals phospholipase A; V.p., Vipera palestinae phospholipase A; DLF, direct lytic factor; PE, phosphatidyl ethanolamine; PS, phosphatidyl serine and PC, phosphatidyl choline.

5. Electron microscopical study of neurotoxic effects of Echis coloratus venom in mice

Neurological disturbances are a prominent feature in experimental animals inoculated with the venom of Echis coloratus (1,2). Studying the neurotoxic actions of this venom, Sandbank and Djaldetti (2) have demonstrated massive penetration of intracardially injected trypan blue and of fluorescite into the brains of treated guinea pigs. Microscopical changes in these brains, revealed by histochemical methods, included appearance of lysosomes and cytolysomes in neurons, appearance of lysosomes in dendrites and axons and appearance of diffuse ATPase in the vicinity of blood vessels. All changes are assumed to be further evidence for an impaired blood brain barrier.

In this study effects of Echis coloratus venom on the permeability of cerebral capillaries to peroxidase and on the ultrastructure of the brain cortex have been examined, using electron microscopy.

Methods

Treatment of animals. Locally bred albino mice, 32-35 gr, were injected intravenously with 10 mg of horseradish peroxidase. Fifteen minutes later Echis coloratus venom, 60 µg, was administered by the same route. Neurological disturbances, such as already described (1,2) were observed. The mice were sacrificed 15 minutes

after envenomation, close to their death.

Preparations of sections from brain cortex for electron microscopy

Brain cortex was fixed in a cold 4% glutaraldehyde solution followed by extensive washing in buffered sucrose (5%). The cortex was cut into thin sections on a freezing microtome. Part of the sections were incubated at room temperature for the peroxidase reaction as described by Graham and Karnovsky (3). All the sections were then post-fixed in 2% OsO_4, dehydrated in graded alcohols, embedded in Epon 812 and cut into ultrathin sections. Sections which did not undergo the peroxidase reaction were stained with uranyl acetate and lead citrate. The preparations were examined in a Phillips 300 electron microscope.

Results and discussion

Sections of brain cortex following peroxidase reaction

A strong positive peroxidase reaction was observed in the blood plasma and in erythrocytes within the lumen of the capillaries. Diffused peroxidase reaction occurred in the cytoplasm of the endothelial cells lining the capillaries (Fig. 5). In some endothelial cells peroxidase positive droplets were observed. The junction between the endothelial cells did not show any peroxidase positive material. A few peroxidase droplets were

observed in the pericapillary space; it is suggested that they passed through the capillary wall. No peroxidase positive material was observed in the neurons.

Sections of brain cortex stained with lead citrate and uranyl acetate.

Multiple membrane-bound vesicles of different sizes were observed within the endothelial cells lining the capillaries (Fig. 6). They were eventually pinocytotic vesicles probably identical with the peroxidase-positive droplets mentioned above. The junctions between the endothelial cells seemed unaffected. In the basal membrane of the capillaries multiple electron-lucent cyst-like areas were observed (Fig. 7,8). Glia cells and neurons appeared normal.

The most noticeable change induced in the mouse brain by Echis coloratus venom is increased capillary permeability. Such change has been shown in some cases to result from separation of interendothelial junctions (4,5). In our preparations, however, these seemed unaffected, while numerous vesicles appeared within the endothelial cells lining the capillaries. We suggest that the mechanism underlying increased permeability is enhanced pinocytotic activity of these cells. A similar type of endothelial damage has been observed in rats and rabbits on administration of Vipera palestinae hemorrhagin (6).

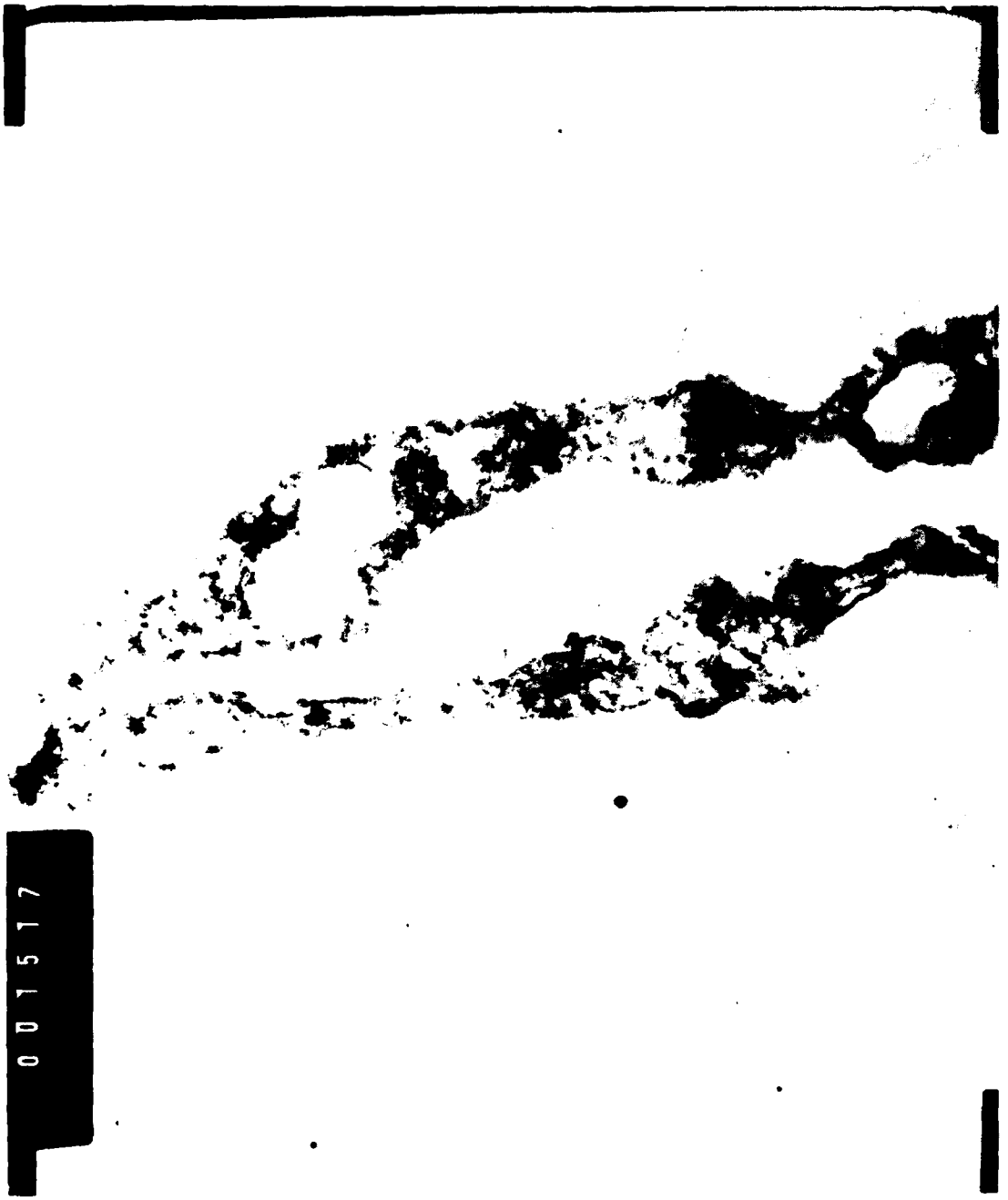
Figures

Fig. 5. Peroxidase droplets in cerebral capillary endothelial cells. Small amounts of peroxidase positive material also outside capillary wall. Magnification X 44,000 (P=22,000).

Fig. 6. Cerebral capillary. Endothelial cytoplasm swollen containing multiple pinocytotic vesicles. Magnification X 36,000 (P=18,000).

Fig. 7. Cerebral capillary. Multiple pinocytotic vesicles in cytoplasm of endothelial cells. Multiple cystic electron lucent areas in basal membrane. Magnification X 36,000 (P=18,000).

Fig. 8. Multiple cystic lesions in basal membrane of cerebral capillary. Magnification X 44,000 (P=22,000).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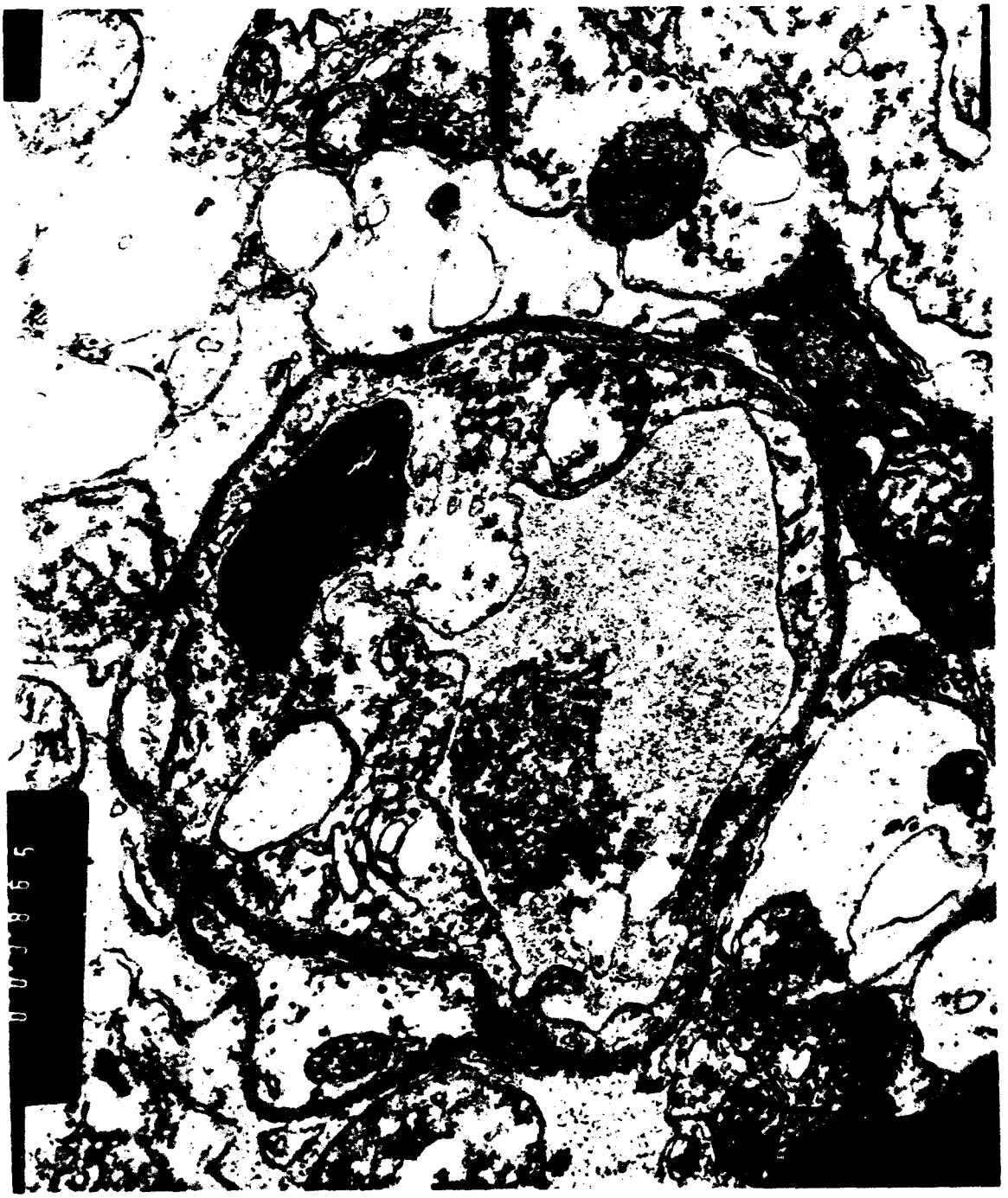

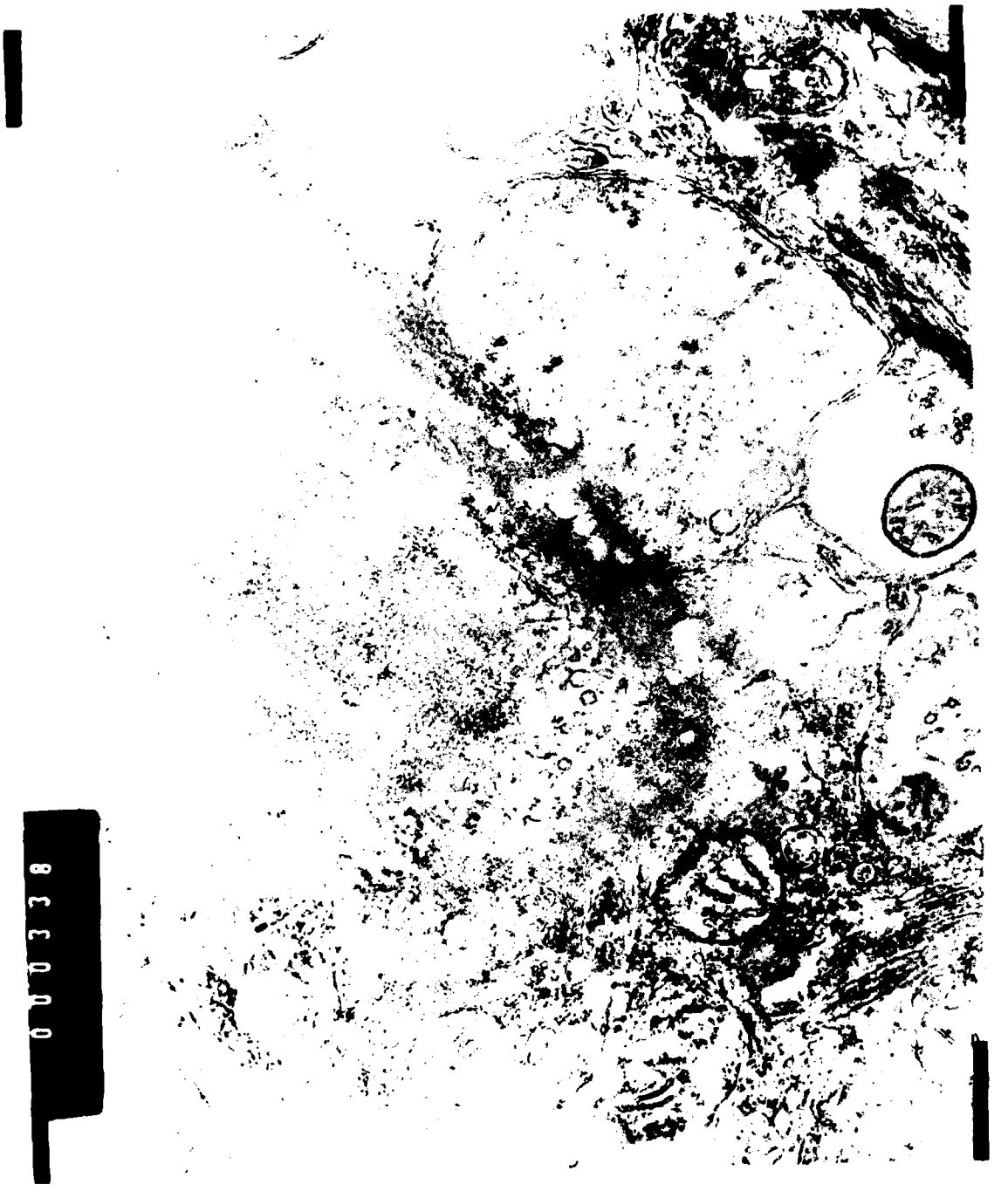
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General conclusions and comments

The studies reported concern in vivo effects of snake venoms and of toxins isolated from them, as well as in vitro experimentation directed at elucidation of the mode of action of some of these toxins. The main results and the conclusions derived from them are as follows.

1. Hemorrhagins isolated from the venoms of Vipera palestinae and Echis coloratus are homogenous by current methods for protein analysis. Both hemorrhagins possess hemorrhagic as well as proteolytic activities. In Vipera palestinae hemorrhagin these activities can be separated by treatment with specific antiserum, which precipitates the hemorrhagic activity only. This activity, however, could not be regained from the immune precipitate. Comparison of the two hemorrhagins revealed partial identity of their antigenic determinants and partial cross neutralization of their lethal activity by heterologous antiserum. These findings suggest that the two hemorrhagins have identical structure at certain parts of the molecule, possibly at the active site. A detailed chemical analysis of the two pure hemorrhagins is needed to test the correctness of this suggestion.

2. Cobra phospholipase A, known to interact with phospholipids in various biological membranes, has now been shown to hydrolyze phospholipids in sialic acid-depleted red blood cells and in mouse brain slices as well. Analysis of phospholipase-treated red blood

cells demonstrates that the negative charges on red cell membranes, contributed by sialic acid and sialoproteins, do not affect the interaction of phospholipase A with membranal phospholipids. Loss of phospholipids from brain slices markedly impairs influx of histidine and of other amino acids into them. It follows that the saturable membranal system for amino acid transport is dependent on phospholipid.

A study of the interaction of phospholipase A with a purified lecithin substrate resulted in the finding that under appropriate conditions it hydrolyzed the lysolecithin product to yield fatty acid and glyceryl phosphorylcholine. Lysolecithin hydrolysis by phospholipase A is best achieved at alkaline pH. Pure phospholipases A from Naja naja and from Vipera palestinae venom hydrolyze lysolecithin with comparable efficiency under optimal conditions of pH and substrate concentration. Their activity towards lecithin however is different, Naja naja phospholipase A being the more active enzyme. The difference in activity of the two phospholipases towards lecithin only is interpreted to depend on the state of this substrate in aqueous solution and may therefore reflect a difference in molecular shape.

3. A direct lytic factor from Ringhals venom, known to interact with various unmodified biological membranes rendering their phospholipid constituents available to the action of phospholipases A, has

now been shown to affect equally well erythrocytes and ghosts depleted of sialic acid or sialoproteins. Removal of the latter negatively charged constituents from cells did not change the phospholipid content of their membranes. As the lytic factor is a basic protein and assumed to directly interact with negative surface charges, the possibility of its binding to sialic acid is now ruled out. Identification of a membrane constituent responsible for attachment of the lytic protein requires further research in this field.

4. Echis coloratus venom, known to cause neurological disturbances in experimental animals and to impair the blood-brain barrier for various molecules, has now been shown to damage brain capillary endothelial cells. Brains from envenomated mice, examined by electron microscopy, contain numerous vesicles within the endothelial cells. Peroxidase, intravenously administered into envenomated mice, has appeared within the same cells as diffuse matter or in the form of droplets. As the interendothelial junctions seemed unaffected, it is suggested that a venom neurotoxic factor acts on the capillary endothelial cells and enhances their pinocytotic activity, thus rendering the capilleries permeable to large molecules. Further studies in this field would call for isolation of a constituent of Echis coloratus venom which specifically induces these changes in brain.

The studies described reflect further progress towards better understanding of the actions of venoms and of toxins isolated from them.

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